Down regulation effect of *Rosmarinus officinalis* polyphenols on cellular stress proteins in rat pheochromocytoma PC12 cells

Abdelfatteh EL Omri · Junkyu Han · Manef Ben Abdrabbah · Hiroko Isoda

Abdelfatteh EL Omri · Junkyu Han · Hiroko Isoda (address)

Graduate School of Life and Environmental Sciences, Alliance for Research on North Africa (ARENA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan.
isoda.hiroko.ga@u.tsukuba.ac.jp

Manef Ben Abdrabbah (address)

Unité de Recherche Physico-chimie et Moléculaire: Institut Préparatoire aux Etudes Scientifiques et Techniques IPEST, La Marsa, 7 November Carthage University 2070 Tunis - Tunisia.

*Corresponding author. Alliance for Research on North Africa (ARENA), University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki 305-8572, Japan.Tel: 81-29-853-5775; Fax: +81-29-853-5776.
e-mail adress: isoda@sakura.cc.tsukuba.ac.jp (H. Isoda)
A. El Omri
omriabdel@gmail.com

Abstract

Polyphenols are known to exhibit wide spectrum of benefit for brain health and to protect from several neurodegenerative diseases. The present study was sought to determine the neuroprotective effects of *Rosmarinus officinalis*' polyphenols (luteolin, carnosic acid, and rosmarinic acid) through the investigation of stress-related proteins. We carried out measurement of the expression of heat-shock protein (Hsp) 47 promoter in heat stressed Chinese hamster ovary transfected cells. We performed proteomic and confirmed gene expression by real time PCR analysis in PC12 cells. Results showed that these compounds modulated significant and different effects on the expression of 4 stress-related proteins: heat shock protein 90 α (*Hsp90*), Transitional endoplasmic reticulum ATPase (*VCP/p97*), Nucleoside diphosphate kinase (*NDK*), and Hypoxia up-regulated protein 1 (*HYOU1*)) at translational and post translational levels in PC12 cells and they downregulated the expression of *Hsp47* activity in Chinese hamster transformed cells. These findings suggest that luteolin, carnosic acid, and rosmarinic acid may modulate the neuroprotective defense system against cellular stress insults and increase neuro-thermotolerance.

Key words

Rosmarinus officinalis

Hsp90 α

VCP/p97

NDK

HYOU1

Hsp47

INTRODUCTION

Neurodegenerative disease manifests in elderly people and most commonly in developed countries where life span is long. However, the World Health Organization has recognized it as a global problem since it is the fourth most common source of death. Neurodegenerative diseases are expected to impose severe impact on our society emotionally, socially and financially by the next coming decades (Park et al. 2010). In this respect understanding, the pathogenesis mechanism and finding potential therapeutic targets for such diseases is becoming a focus point for the scientific community.

In neuronal cells, heat shock proteins (Hsps), provide a fundamental mechanism to defend the cell against external diverse physiological stress (Luo et al. 2007). Induced by several stressors like temperature, hypoxia, inflammation, infections and environmental pollutants, stress proteins (Hsps) play key roles in living systems (Taguchi et al. 2007). It was postulated by several studies that Hsps are working as chaperones together with ubiquitin-proteasome system (UPS) to assist folding/refolding of nonnative protein, to help in the degradation of irreversibly damaged proteins, and other proteins essential for the protection and recovery from cell damages associated with perturbation of protein homeostasis. In neuronal cells, Hsps may have anti-apoptotic effects and keep the homeostasis against stress conditions (Chen and Brown 2007; Luo et al. 2007; Winklhofer et al. 2008; Oza et al. 2008). Recently, Hsps became a research therapeutic target in neurodegenerative disorder and aging because the pathogenesis mechanism of these diseases is thought to be related to an abnormal increase of Unfolded Protein Response (UPR), failure of UPS and protein misfolding and/or aggregation (Zhao et al. 2010).

Numerous studies in the last decade have shown that dietary polyphenols may have, *in vitro* and *in vivo*, a neurorescue impact in aging and neurodegenerative diseases to retard or even reverse the accelerated rate of neuronal degeneration (Rasmassy 2006, Sun et al. 2010, Rajeswari

and Sabesan 201; Ortega 2006; Spencer 2009) and aging (Queen and ; Wilson et al. 2006). However, little is done about their effect on Hsps in relation with neurodegenerative disease.

R. officinalis is traditionally used to improve memory, in connection with AD and dementia, for general symptoms of old age, debility and fatigue (El Omri et al. 2010). Recently several studies showed that *R. officinalis* or its main compounds like carnosic acid (CA), rosmarinic acid (RA) and luteolin (Lut) (Kosaka et al. 2010, El Omri et al. 2010 and Lin et al. 2010) can be good candidates to substitute nerve growth factor (NGF). Moreover, this plant and its main active compounds have been reported to be anti Alzheimer's disease AD (Liu et al. 2010, Lin et al. 2010 and El omri et al. 2010) anti Parkinson's disease (PD) (Park et al. 2010) and anti amyotrophic lateral sclerosis (ALS) (Shimojo et al. 2009).

This study is the first conducted to determine the effects of *R.officinalis'* polyphenols: Lut, CA, and RA on stress-related proteins expression in PC12 cells and to confirm their expression using RT-PCR.

MATERIALS AND METHOD

Chemicals

The following reagents were purchased from several manufacturers and were used to prepare the culture medium and the required solutions: Dulbecco's modified Eagle medium (DMEM) was purchased from Sigma Aldrich (United kingdom), fetal bovine serum (FBS) was purchased from Bio west (France), horse serum (HS) and Geneticin (G418) were purchased from Invitrogen (Carlsbad, CA, USA), F12 Medium was from Invitrogen (Tokyo, Japan). Penicillin-streptomycin was purchased from Lonza,Walkersville Inc., (MD, USA), DTT and TEMED were purchased

from Amersham Bioscience (Sweden). Luteolin, carnosic acid, NGF 7s, Trizma base, kanamycin solution, trypsin (ethylenediaminetetra-acetic acid [EDTA]), $MgCl_26H_2O$, 4-methylumbelliferyl-β-galactose (MUG), protease inhibitor cocktail, and Ribonuclease A, all were purchased from Sigma-Aldrich (USA). Rosmarinic acid, and $Na_2HPO_47H_2O$ were purchased from MP Biomedicals LLC (France). Spemine base and acetonitrile were purchased from Sigma-Aldrich (Germany). Thiourea, APS and protein rainbow marker were purchased from GE Healthcare (United Kingdom), urea, acrylamide, Bis, Bromophenol blue, CHAPS, CBB G-250, Glycol, glycine, iodoacetamide, SDS, Tris, urea, IPG buffer and IPG strips were purchased from GE Healthcare (Sweden). Lysis buffer was from (Promega). NaCl, KH2PO4, KCl, NaH2PO42H2O, bovine serum albumin, NaN_3, dimethyl sulfoxide, glycine, NaOH, and Deoxyribonuclease DNAse A were purchased from Wako (Japan).

Cell culture

Chinese hamster ovary (CHO) cells stably transfected with (+) or without (-) an Hsp 47 promoter were used for this experiment (Isoda et al. 2004). The cells were provided by S. Yokota (Kaneka), and were grown as adherent monolayer in 75 cm2 tissue culture flasks using F12 Medium supplemented with 10% Fetal Bovine Serum, 200 μg/mL of G418 (Gibco BRL 13075-015) and 0.1 g/L kanamycin solution. The cultures were maintained in a 5% CO_2 incubator at 37 °C. Cell passage was carried out at 80% confluence at 1:2 ratio using 0.25% trypsin with 1 mM EDTA.

Hsp47-transformed cells were grown as adherent monolayer in 75 cm2 tissue culture flasks using F12 Medium supplemented with 10% Fetal Bovine Serum, 200 μg/ml of and 0.1 g/L kanamycin

solution. The cultures were maintained in a 5% CO_2 incubator at 37 °C. Cell passage was carried out at 80% confluence at 1:2 ratio using 0.25% trypsin with 1 mM EDTA. The cells were used between passage 3 and 8 for the reported experiments.

PC12 cells (Riken Tsukuba, Japan) were cultured in 75 cm^2 flask (BD Falcon, USA) and maintained in DMEM containing 10% heat inactivated horse serum and 5% fetal bovine serum supplemented with 100 U/mL penicillin and 100 µg/ml streptomycin, in a water-saturated 5% CO_2 atmosphere at 37°C. The cells were used between passage 3 and 8 for the reported experiments.

Heat shock protein 47 assay

Hsp47-transformed cells were trypsinized and plated onto 96-well plates at initial concentrations of 1×10^4 cells per well in 100 µL of culture medium. The cells were allowed to attach for 48 h at 37 °C supplemented with 5% CO_2, heat-shocked for 90 min at 42˚C, 5% CO_2 and recovered for 2 h in a 5% CO_2 incubator. Then, medium was removed and changed by 100 µL of samples diluted with medium at desired concentrations and incubated with cells for 3h in 5% CO_2 incubator at 37 °C.

After incubating cells with samples, the medium was removed and the cells washed twice with PBS. 50 µL lysis buffer (Promega) was then added and the plates incubated for 30 min at room tremperature. 20 µL of cell lysate was transferred to a new plate, to which 100 µL of substrate solution (10 mM $NaH_2PO_4•_2H_2O$, 100 mM NaCl, 1% BSA, 0.005% NaN_3, 1 mM $MgCl_2•6H_2O$, 1% 4-methylumbelliferyl-β galactosidase (MUG), pH 7) was added in order to trigger the conversion of MUG into galactose and methylumbelliferyl by galactosidase. After allowing the reaction to occur in the dark for 30 min at room temperature, 60 µl of reaction stop

buffer (1 M glycine-NaOH, pH 10.3) was added and the fluorescence at 365 nm excitation/ 450 nm emission was then determined using a multi-detection microplate.

PC12 cells treatment and protein extraction

PC12 cells were seeded 2 x10^6 cells/100-mm poly-L-lysine coated dishes (Wako, Japan). Following overnight incubation in a 5% CO_2 humidified incubator at 37˚C. Cells were treated with 50 µM luteolin, 15 µM rosmarinic acid, 20 µM canosic acid and 50 ng NGF for 48 h. The cells were rinsed three times with ice-cold PBS, scraped gently and collected in PBS. Then, the cell pellet was lysed in 1 mL of lysis buffer containing 7 M urea, 2 M thiourea, 4% w/v CHAPS, 1 mM EDTA, 100 mM DTT, 25 mM spermine base, 1% protesase inhibitor cocktail (Han et al. 2010) and 0.1 volume of DNAse I (1mg/mL)/RNAse (0.25 mg/mL) mixture . DNAse I, RNAse, DTT and Protease inhibitor cocktail were immediately added to the extraction-lysis buffer. The extraction was carried out firstly at 4°C for 45 min to degrade nucleic acid then followed by 1 h at room temperature with rotation (Yang et al. 2006). Then the lysate was clarified by ultracentrifugation at 46.000 rpm at 15°C for 60 min. The final protein amount was determined using 2-D Quant kit.

Two-dimensional gel electrophoresis (2-DE)

The first dimension electrophoresis was carried out on an Ettan IPGphor II (GE Healthcare) apparatus. Immobilized pH gradient (IPG) strips (pH 3-10, 24 cm, GE Healthcare) were rehydrated (7 M Urea, 2 M Thiourea, 2 % CHAPS, traces of Bromophenol blue, 50 mM DTT and 0.5 % IPG buffer, IPG buffer and DTT were added immediately before use) with 350 µg of sample solution. The total volume loaded by strip was 450 µL. the rehydration and separation programs were processed using the following parameters: step 1: 500 Vhr, step 2: 750Vhr, step

3: 16.5 KVhr, step 4: 27.5 KVhr and step 5 was 500 v for 24 h. The proteins were separated according to their isoelectric points. The isoelectrically focused IPG strips were immediately equilibrated for 2x 15 min using equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30 % glycerol (w/w), 2 % (w/v) SDS, traces of bromophenol blue). The first equilibration was with 1.0% w/v DTT followed by a second equilibration with 2.5% w/v iodacetamide. Then the strips were immersed in 10 ml of electrophoresis buffer for 5 min.

The strips were subsequently subjected to a second dimension SDS gel (255 mm x 200 mm x 1 mm), the protein were separated using 12% SDS PAGE, using Ettan DALTSix™ electrophoresis unit (GE Healthcare). The SDS-PAGE was performed at 2 w/gel for 40 min, then 15 w/gel until the dye front reached the bottom of gels. After being fixed with 3% ethanol, 0.5% acetate solution, gels were stained by CBB for 8 h. After being distained by rinsing with fixing solution, gels were scanned at 300 dpi resolution and the image were analyzed with Image Master™ 2D software (ver. 4.9: GE Healthcare). For statistical quantification of expression difference with software, three experiments were performed for each treatment. Coomassie blue stained 2-DE gel images were acquired with image scanner and subsequently subjected to visual assessment to detect changes in protein expression level between different treatments. Spots were expressed as percentages (% vol) of relative volumes by integrating the value of each pixel in the spot area as described previously in our study (Han et al. 2010).

In-gel digestion and mass spectrometry

Protein spots of interest were excised from the CBB-stained gel; the excised spots were transferred to Eppendorf tube loaded with 100 µL of 50% ACN/25 mM ammonium bicarbonate solution (1:1). After being distained, gel sample were rehydrated with 100 µL of 100% ACN for

5 min and then thoroughly dried in the speedVac concentrator (miVac, England) for 5 min. Then, the dried gel were reduced in 100 μL 10 mM DTT/25 mM ammonium bicarbonate with shaking at 56°C for 1 h, and washed with 100 μL of 25 mM ammonium bicarbonate with shaking at room temperature for 10 min. Afterward gels particles were alkylated in 100 μL of 55 mM Iocetamide/25 mM ammonium bicarbonate and incubated on dark for 45 min at room temperature and washed as described previously. After that, gel sample were dehydrated with 100 μL of 100% ACN for 10 min and then thoroughly dried in the speedVac concentrator for 5 min. Subsequently the dried were rehydrated with 2 μL/ sample trypsin in 25 mM ammonium bicarbonate with about 1:50 enzyme amount ratio to protein after staying at 4°C for 30 min, and incubated at 37°C for 15 h. After trypsin digestion, the supernatant was transferred to another tube. Then, the remained peptide mixture was extracted twice with 50% ACN/5% formic acid at 37°C for 30 min using 50 μL of the extraction solution for the first time and 25 μL for the second time. Subsequently the combined solution was concentrated in the speedVac to 10 μL and analyzed using MALDI TOF as described in our previous study (Han et al. 2010).

Analysis of gene expression by quantitative Real-time PCR

To confirm the activation of stress-related proteins in *R.officinalis*'polyphenols-treated PC12 cells, the expression of *Hsp90α*, *HYOU1*, *VCP/p97*, and *NDK* were determined by real-time PCR using glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as an internal positive control. After incubating seeded plates for 6 h and 12 h, total RNA was purified using the ISOGEN kit (Nippon GeneCo. Ltd., Japan) following the manufacturer's instructions. Total RNA was quantified by measurement with Thermo scientific nanodrop 2000 (USA). Reverse transcription

reactions were carried out with the Superscript III reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA, using 1 µg of total RNA. Briefly, RNA was denatured at 65 °C for 5 min. and incubated with 1 µL oligo (dT) $_{12–15}$ primers and chilled at 4 °C. After adding SuperScript II reverse transcriptase (200 U) the reaction mix was incubated at 42 °C for 60 min, then 10 min at 70 °C (Han et al., 2010). All Primer sets and TaqMan probes for experimental genes were from Applied Biosystems *Hsp90α* (Rn00822023), *GAPDH* (Rn99999916_s1), were inventoried gene expression assays, *VCP/p97* (Rn01439521_m1), *NDK* (Rn01465378-gH), and *HYOU1* (Rn02586251_m1) were obtained as 'Assays-on-demand' kits.

For the quantification of mRNA, TaqMan real-time quantitative PCR amplification reactions were carried out in an AB 7500 fast real-time system (Applied Biosystems). Amplifications were performed in 20 µL final volume, using 10 µL of TaqMan Universal PCR Master Mix UNG (2X), 1 µL of the corresponding primer/probe mix and 9 µl of template cDNA (70 ng μL^{-1}). Cycling conditions were: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 95°C for 15 s followed by 60°C for 1 min.

RESULTS

Luteolin, carnosic acid and rosmarinic acid decrease the expression of Heat shock protein 47.

Hsp47-transformed cells were heat-shocked for 90 min at 42˚C, 5%CO_2 and recovered for 2 h, and then we proceed to the screening of *R. officinalis*'EtOH extract and its main polyphenols (lut, CA, and RA) effect on the recovery of *Hsp47*. As shown in Fig.1A, 70% EtOH extract of *R. officinalis* reduced significantly the expression of *Hsp47* at 1/100 dilution v/v, by 25 %. Additionally, Lut, CA, and RA expressed the same activities. As indicated in Fig.1B, all 3

compounds significantly reduced the expression of *Hsp47* in a dose dependent manner. At high doses of Lut, CA, and RA there were a recovery of 20% of control.

Effect of luteolin, carnosic acid and rosmarinic acid on stress related protein expression

Since the major phenolic compounds of *R. officinalis* are Lut, CA, and RA, in subsequent analysis we assumed that this anti-stress activity could be mainly due to the presence of these compounds and tried to elucidate their neuroprotective effect in PC12 cells using proteomics analysis.

PC12 cells were treated with Lut (50 μM), CA (20 μM), and RA (15 μM) for 48 h, then total proteins were separated by 2D-gel electrophoresis. A protein pattern of PC12 cells is shown in Fig. 2A. Approximately 200 well-resolved spots were detected in each coomassie blue-stained gel, with molecular-mass ranges of 15–225 kDa and a *pI* ranging from 3 to 10. Several spot 'volumes from treated-PC12 cells were changed. As shown in Fig.2B, treatment with *R. officinalis* polyphenols caused substantial change in 4 particular spots ($P < 0.05$). Protein spots were selected and analyzed with MALDI-TOF. The peptide mass fingerprinting (PMF) spectra was used to search protein database using Mascot engine versus Swiss prot database as reported in our previous study (Han et al. 2010). These spots were identified to be stress-related proteins: *Hsp90*, *VCP/p97*, *HYOU1*, and *NDK* (Table 1). Lut and CA significantly down regulated all 4 proteins: Lut treatment induced a decrease by around 40 % of studied proteins, CA induced severe down regulation of *Hsp90* and *VCP/p97* However, RA decreased significantly only the expression of *HYOU1* and *VCP/p97* to 0.4 and 0.7 as % of Ctrl. Spot volume.

Validation of differentially expressed stress related protein by quantitative RT-PCR.

To confirm the protein expression of *Hsp90*, *HYOU1*, *VCP/p97*, and *NDK*, we evaluated mRNA expression of previously cited proteins after 6 h and 12 h treatment. As shown in Fig. 3A, CA, Lut and NGF treatments respectively significantly reduced *Hsp90* mRNA expression to 0.2, 0.84 and 0.25. Lut decreased *Hsp90* expression from 1.5 (6 h treatment) to 0.81 (12 h treatment). Meanwhile, CA and NGF increased *Hsp90* after 12 h to 0.80 and 0.88, respectively. RA showed significant high expression of *Hsp90* at 6 h which was decreased to control level after 12 h treatment. *VCP/p97* was significantly down regulated by CA, RA, and NGF to 0.23, 0.64, and 0.15 respectively after 6 h treatment. However Lut treatment only showed decrease in mRNA expression after 12 h (Fig. 3B). Similarly, *HYOU1* was down regulated by CA, RA, and NGF to 0.16, 0.65, and 0.31 respectively. Lut treatment showed an increase after 6 h to 1.4 fold and a decrease to 0.73 fold after 12 h (Fig. 3C).

NDPK mRNA expression was decreased by all treatments. Lut, CA, RA and NGF showed a significant decrease in *NDK* expression after 6 h treatment to 0.77, 0.58, 0.47, and 0.13. This effect was maintained in case of Lut and CA and it was increased to 1.17 and 1.11 respectively for NGF and RA after 12 h treatment.

Discussion

Previously, we and others demonstrated that the main polyphenols of *R.officinalis*: Lut, CA, and RA are able to induce PC12 cells differentiation (El Omri et al. 2010; Lin et al. 2010). In the present study we observed for the first time that Lut, CA, and RA reduced significantly the

expression of *Hsp47* in stress- heated Chinese hamster ovary transfected cells (Fig. 1). Proteomic analysis and qRT-PCR showed that differentiation of PC12 cells into neuron-like cells is associated with an attenuated stress related proteins. Our result is consistent with previous observations of a reduced induction of Hsp70 (Dwyer et al. 1996), NDPK (Kim et al. 2007), and *VCP/p97* (Kobayashi et al. 2002) during neuronal differentiation of PC12 cells (Dwyer et al. 1996).

Hsp47 is a heat stress protein that interacts with procollagen in the lumen of the endoplasmic reticulum (ER) (Taguchi et al. 2007). It is the main chaperone involved in collagen elaboration and matutration (Rocnik et al. 2002). Reducing the activity of *Hsp47* means that R. officinalis polyphenols could protect mammalian cells against heat stress and increase thermotolerance. *Hsp90* is a molecular chaperone. In neurodegenerative diseases, it is involved in the protection of neuronal cells against the accumulation of toxic aggregates (Luo et al. 2010). *VCP/p97* is cytosolic chaperone required for Endoplasmic Reticulum-Associated Protein Degradation (ERAD). It is involved in a variety of cellular processes, including membrane fusion and ubiquitin-dependent protein degradation and it is chaperone-like protein (Nagahama et al. 2002). Recent studies showed that inhibition or stable complexing of *Hsp90* may alleviate and prevent from some neurological disease with motor impairments and Taupathie (Ali et al. 2010). *HYOU1* belongs to Hsp70 superfamily. It has been suggested to be a neuroprotective factor against ischemia and excitotoxicity. It was reported to be upregulated under hypoxic or excitotoxicity conditions, that potentially induce ER stress in neurons (Zhao et al. 2010). In our study, we demonstrated that *R.officinalis* polyphenols downregulated the expression of HYOU1. Subsequently they may alleviate stress insults in PC12 cells through their antioxidant activity. *NDK* is involved in the proteolytic functions of the proteasome. It may act by

catalyzing the activities of ATP hydrolysis when Hsp70 and *VCP/p97* are activated (Yano et al. 1999). Meanwhile, (Kimura et al. 2002) reported the involvement of NDP kinases in the regulation of cell growth and differentiation. Particularly in PC12 cells, *NDK* may control the molecular switch to determine the cell fate toward proliferation or differentiation in response to environmental signals.

In neuron cells, physiological and pathological processes that disturb protein folding in the ER cause ER stress and activate a set of signaling pathways termed UPR (Samali et al. 2009). Particularly, in neurons misfolded and/or aggregated proteins cannot be diluted, and accumulate with aging (Chen and Brown 2007), leading to several neurodegenerative disorders, like AD, PD, ALS, Huntington's disease (HD), and other polyglutamine expansion disorders (Luo et al. 2007, Oza et al., 2008). It was suggested by Taguchi et al. (2007) that targeting Hsp is a promising alternative in the area of neurodegenerative disorder, where protein aggregation and neuron degeneration are the common pathological features. In this respect, it was demonstrated by Wilson et al. (2006) that blue berry polyphenol uptake increased life span of *C. elegans* by promoting stress resistance. Melatonin was demonstrated by Ozacmak et al. (2006) to protect rats by reducing Hsp70 expression during chronic cerebral hypoperfusion. Resveratrol was reported to protect cells against heat stress through chaperone activation (Putik et al. 2005). Curcumin consumption by Indian reduced AD incidence in comparison to American people (Ali et al. 2010).

As a part of this study, we examined possible mechanisms for the beneficial effects of *R.officinalis'* polyphenols treatment in a neuronal cell-like model. As shown stress induced-protein expression by Lut, CA, and RA was correlated with qRT-PCR. However at mRNA level we observed a decrease after 6 h followed by an increase of mRNA of different gene in case of

CA and RA treatment, and the opposite was observed for Lut treatment. This observable fact could be correlating with structure-functions of these 3 different polyphenols. Apart from being great scavengers of free radicals, *R. officinalis'* polyphenols may directly stimulate the cell defense against stress response through cellular chaperone in early time treatment. As it is known, that antioxidant after scavenging free radical, they became themselves pro-oxidant after being oxidized in cell culture media (Balliwell 2008). Oxidation of polyphenols produces peroxide, hydroperoxide a complex mixture of semiquinones and quinones, all of which are potentially cytotoxic (Balliwell 2008). In response to these pro-oxidant, the cell may act to regulate and conserve its stress defense system to maintain ER function and thus protect cells from toxic insults (Zhang et al. 2007).

Regardless of their effect on downregulation of *Hsp47*, *Hsp90*, *VCP/p97*, *HYOU1*, and *NDK*, it is clear from these experiments that natural polyphenols available in rosemary leaves can reduce neuronal stress, and increase thermotolerance. This is a significant finding that lends support to previous experiments on cultured neuronal cells or *in vivo* studies showing beneficial effects against neurodegenerative-related declines.

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References

Ali YO, Kitay BM, Zhai RG (2010) Dealing with misfolded proteins: examining the neuroprotective role of molecular chaperones in neurodegeneration. Molecules 15:6859-6887

Chen S, Brown IR (2007) Neuronal expression of constitutive heat shock proteins: implications for neurodegenerative diseases. Cell Stress Chaperones 12:51-58

Dwyer DS, Liu Y, Miao S, Bradley RJ (1996) Neuronal differentiation in PC12 cells is accompanied by diminished inducibility of Hsp70 and Hsp 60 in response to heat and ethanol. Neurochemical Res 21:659–666

El Omri A, Han J, Yamada P, Kawada K, Abdrabbah MB, Isoda H (2010) *Rosmarinus officinalis* polyphenols activate cholinergic activities in PC12 cells through phosphorylation of ERK1/2. J Ethnopharmacol 131:451-458

Halliwell B (2008) Are polyphenols antioxidants or pro-oxidants? What do we learn from cell culture and in vivo studies? Arch Biochem Biophys 476:107-112.

Hoffrogge R, Beyer S, Völker U, Uhrmacher AM, Rolfs A (2006) 2-DE proteomic profiling of neuronal stem cells. Neurodegener Dis 3:112-121

Isoda H, Koyama T, Tasaki M, Oka S, Sugiura N, Maekawa T, Inamori Y, Yokota S, Kitahara M, Nagata K (2004) High-sensitive detection of environmental pollutants. US Patent 6740521 B2.

Kim SH, Fountoulakis M, Cairns NJ, Lubec G. (2002) Human brain nucleoside diphosphate kinase activity is decreased in Alzheimer's disease and Down syndrome. Biochem Biophys Res Commun 296 :970-975.

Kimura N, Shimada N, Ishijima Y, Fukuda M, Takagi Y, Ishikawa N (2003) Nucleoside diphosphate kinases in mammalian signal transduction systems: recent development and perspective. J Bioenerg Biomembr 35:41-47

Kobayashi T, Tanaka K, Inoue K, Kakizuka A (2002) Functional ATPase activity of p97/valosin-containing protein (VCP) is required for the quality control of endoplasmic reticulum in neuronally differentiated mammalian PC12 cells. J Biol Chem 277:47358-47365

Kosaka, K, Mimura J, Itoh K, Satoh T, Shimojo Y, Kitajima C, Maruyama A, Yamamoto M, Shirasawa T (2010) Role of Nrf2 and p62/ZIP in the neurite outgrowth by carnosic acid in PC12h cells. J Biochem. 147:73-81

Lin CW, Wu MJ, Liu IY, Su JD, Yen JH (2010) Neurotrophic and cytoprotective action of luteolin in PC12 cells through ERK-dependent induction of Nrf2-driven HO-1 expression. J Agric Food Chem 58: 4477-4486.

Liu R, Gao M, Qiang GF, Zhang TT, Lan X, Ying J, Du GH (2009) The anti-amnesic effect of luteolin against amyloid β_{25-35} peptide induced toxicity in mice involve the protection of the neurovascular unit. Neuroscience 162:1232-1243

Luo W, Sun W, Taldone T, Rodina A, Chiosis G (2010) Heat shock protein 90 in neurodegenerative diseases. Mol Neurodegener 5:24

Nagahama M, Suzuki M, Hamada Y, Hatsuzawa K, Tani K, Yamamoto A, Tagaya M (2003) SVIP is a novel *VCP/p97*-interacting protein whose expression causes cell vacuolation. Mol Biol Cell 14:262-273

Ortega RM (2006) Importance of functional foods in the Mediterranean diet. Public Health Nutr 9:1136-1140

Oza J, Yang J, Chen KY, Liu AY (2008) Changes in the regulation of heat shock gene expression in neuronal cell differentiation**.** Cell Stress Chaperones 13:73-84

Ozacmak VH, Barut F, Ozacmak HS (2009) Melatonin provides neuroprotection by reducing oxidative stress and HSP70 expression during chronic cerebral hypoperfusion in ovariectomized rats. J Pineal Res 47:156-63

Park SE, Kim S, Sapkota K, Kim SJ (2010) Neuroprotective effect of *Rosmarinus officinalis* extract on Human dopaminergic cell line, SH-SY5Y. Cell Mol Neurobiol 30:759-767.

Putics A, Végh EM, Csermely P, Soti C (2008) Resveratrol induces the heat-shock response and protects human cells from severe heat stress. Antioxid Redox Signal. 10:65-75.

Putics A, Végh EM, Csermely P, Soti C (2008) Resveratrol induces the heat-shock response and protects human cells from severe heat stress. Antioxid Redox Signal 10:65-75.

Queen BL, Tollefsbol TO (2010) Polyphenols and aging. Curr Aging Sci 3(1):34-42

Rajeswari A, Sabesan M (2008) Inhibition of monoamine oxidase-B by the polyphenolic compound, curcumin and its metabolite tetrahydrocurcumin, in a model of Parkinson's disease induced by MPTP neurodegeneration in mice. Inflammopharmacology 16:96-9

Ramassamy C (2006) Emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases: a review of their intracellular targets. Eur J Pharmacol 545:51-64

Rocnik EF, van der Veer E, Cao H, Hegele RA, Pickering JG (2002) Functional linkage between the endoplasmic reticulum protein *Hsp47* and procollagen expression in human vascular smooth muscle cells. J Biol Chem 277:38571-38578

Samali A, Fitzgerald U, Deegan S, Gupta S (2010) Methods for monitoring endoplasmic reticulum stress and the unfolded protein response. Int J Cell Biol.doi:10.1155/2010/830307.

Shimojo Y, Kosaka K, Noda Y, Shimizu T, Shirasawa T (2010) Effect of rosmarinic acid in motor dysfunction and life span in a mouse model of familial amyotrophic lateral sclerosis. J Neurosci Res 88:896-904

Spencer, JPE (2009) Flavonoids and brain health: multiple effects underpinned by common mechanisms. Genes Nutr 4:243–250

Sun AY, Wang Q, Simonyi A, Sun GY (2010) Resveratrol as a therapeutic agent for neurodegenerative diseases. Mol Neurobiol 41:375-383

Taguchi T, Razzaque MS (2007) The collagen-specific molecular chaperone *Hsp47*: is there a role in fibrosis? Trends Mol Med 13:45-53

Wilson MA, Shukitt-Hale B, Kalt W, Ingram DK, Joseph JA, Wolkow CA (2006) Blueberry polyphenols increase lifespan and thermotolerance in Caenorhabditis elegans. Aging Cell 5:59-68

Winklhofer KF, Tatzelt J, Haass C (2008) The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. EMBO J 27:336-349

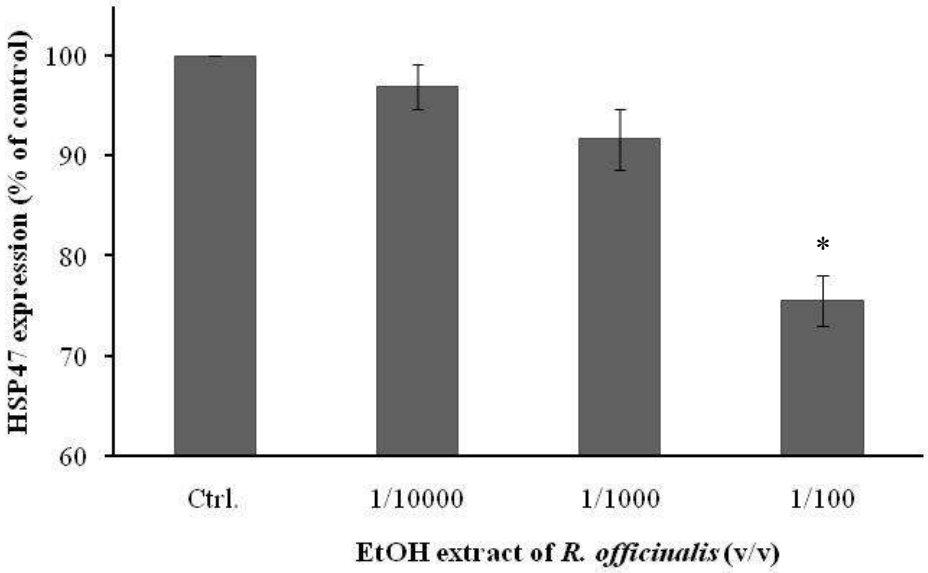
Yano M, Mori S, Kido H (1999) Intrinsic nucleoside diphosphate kinase-like activity is a novel function of the 20 S proteasome. J Biol Chem 274:34375-34382

Zhao L, Rosales C, Seburn K, Ron D, Ackerman SL (2010) Alteration of the unfolded protein response modifies neurodegeneration in a mouse model of Marinesco-Sjögren syndrome. Hum Mol Genet 19:25-35

List of table and figure

Fig. 3 Effect of *R. officinalis* polyphenols on the expressions of *Hsp90, Vcp, HYOU1*, *NDK* mRNAs in PC12 cells. GAPDH was used as a housekeeping gene. The mRNA expression of all gene was normalized by GAPDH mRNA expression and expressed as ratio of Ctrl. PC12 cells were treated with 30 μM luteolin, 20 μM carnosic acid, 15 μM rosmarinic acid and 50 ng/ml NGF for 6 h and 12 h. Each bar represents the mean ± SD of three independent experiments. **P* < 0.05 treatment vs. control (Student's t-test).

A


HSP47 expression (% of control)
100
90
80
70
60
*
Ctrl.
1/10000
1/1000
1/100
EtOH extract of R. officinalis (v/v)

B

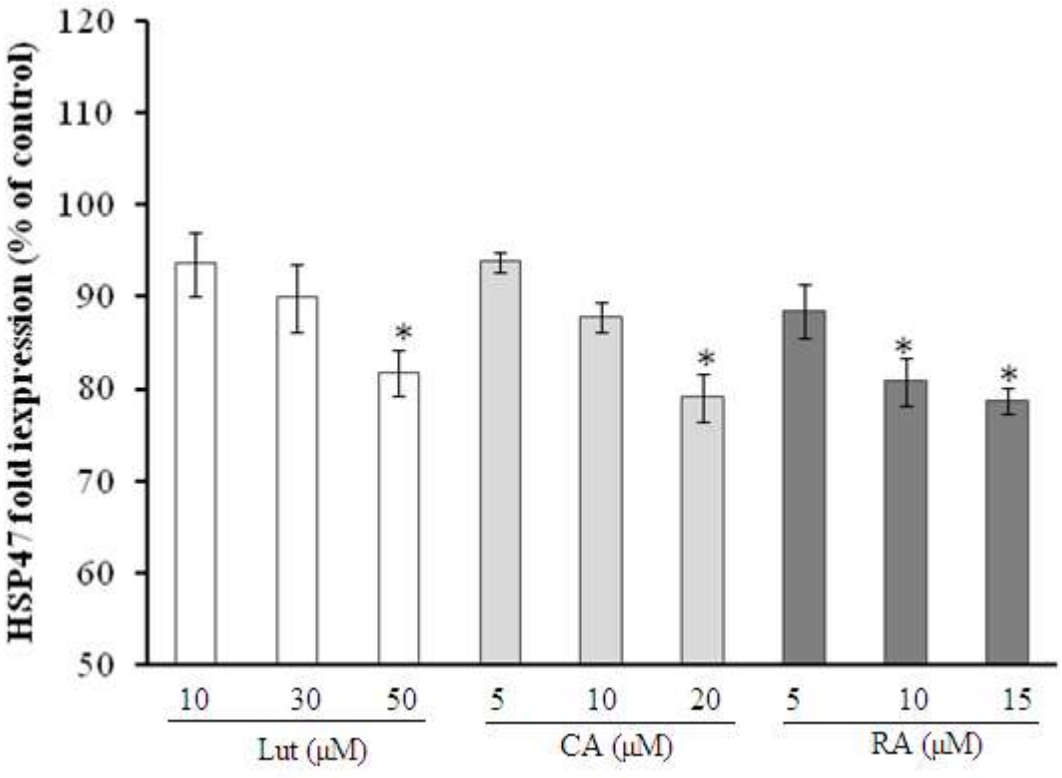

HSP47 fold iexpression (% of control)
120
110
100
90
80
70
60
50
*
*
*
*
*
10
30
50
5
10
20
5
10
15
Lut (μM)
CA (μM)
RA (μM)

Fig. 1

A

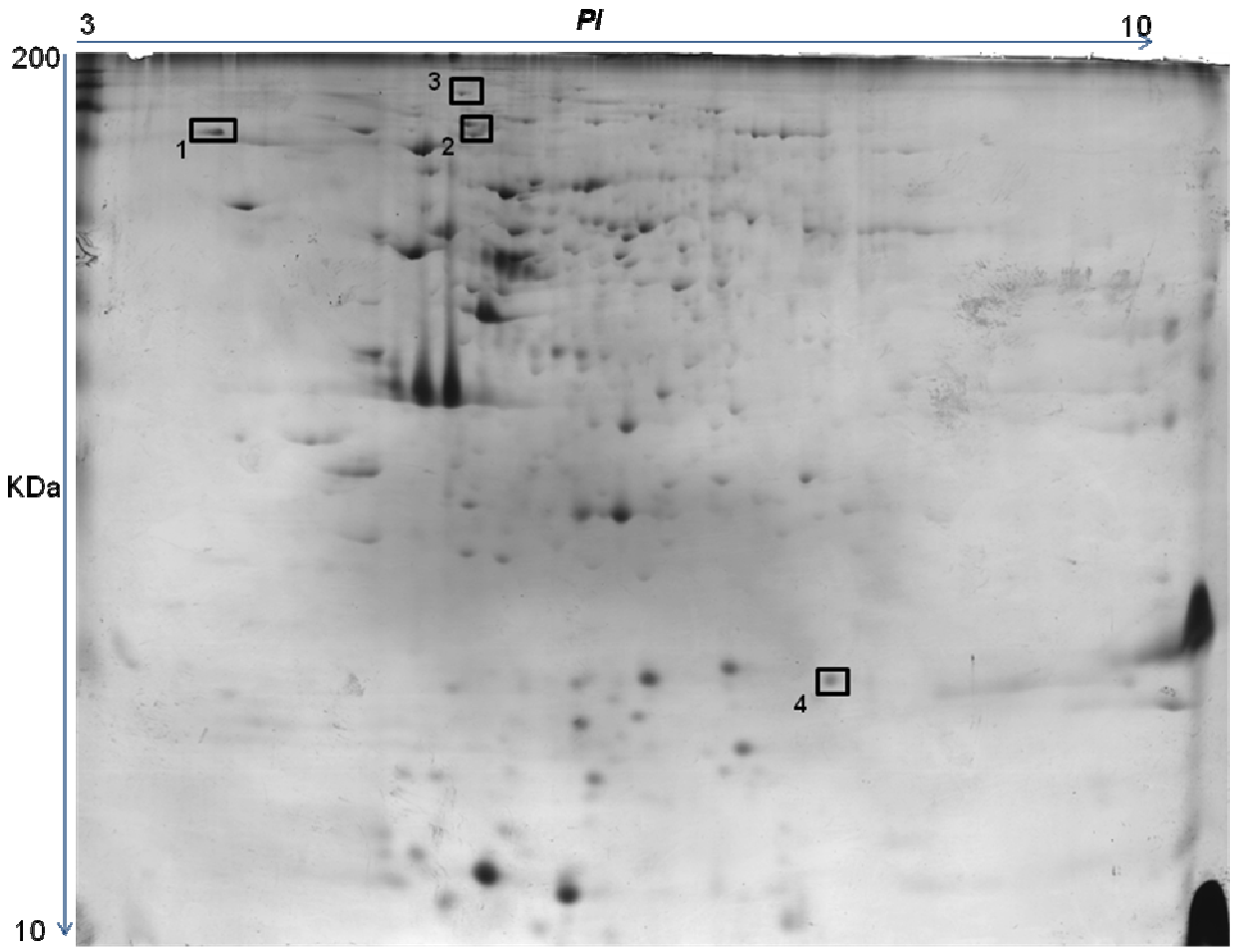


Fig. 2 A

B

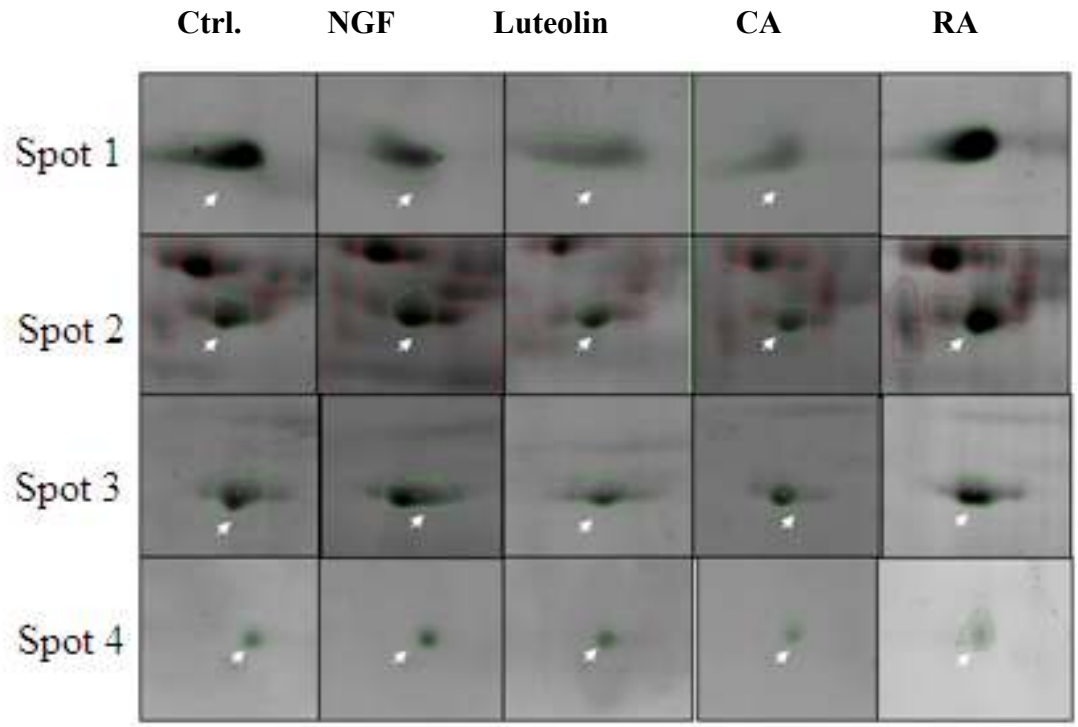


Fig. 2 B

C

.

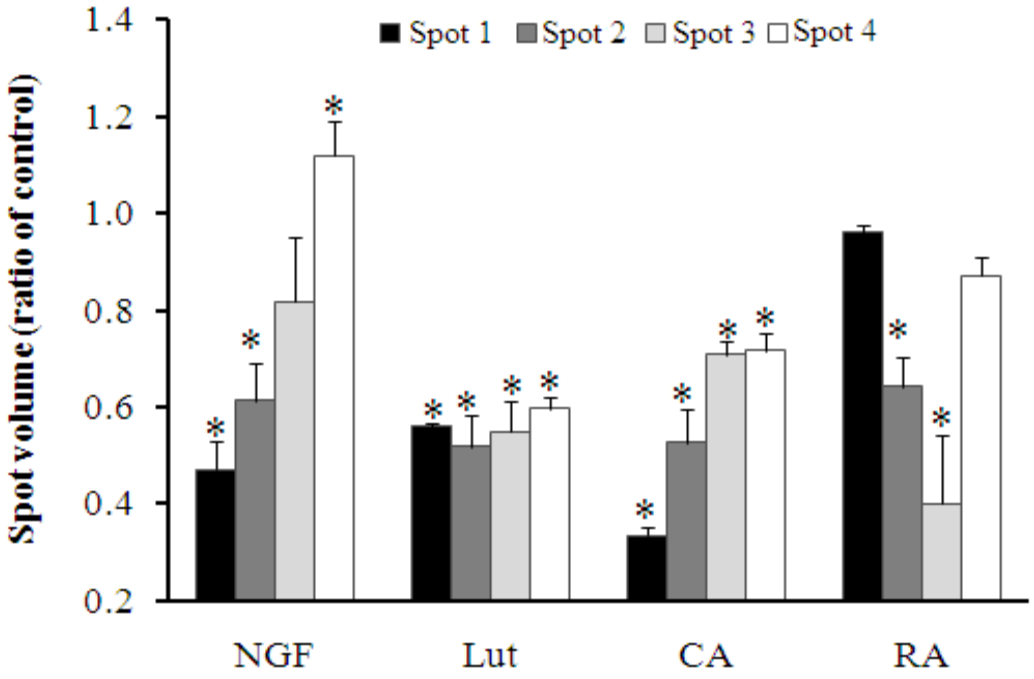
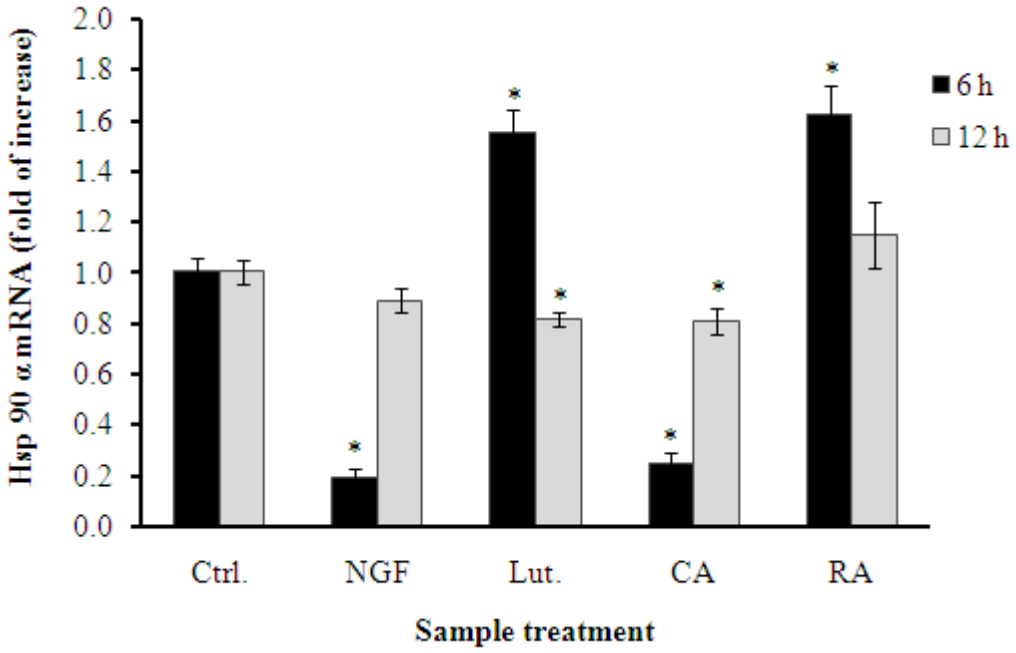

Spot 1
Spot 2
Spot 3
Spot 4
Spot volume (ratio of control)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
NGF
Lut
CA
RA

Fig. 2 C

A


Hsp 90 α mRNA (fold of increase)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6 h
12 h
Ctrl.
NGF
Lut.
CA
RA
Sample treatment

B

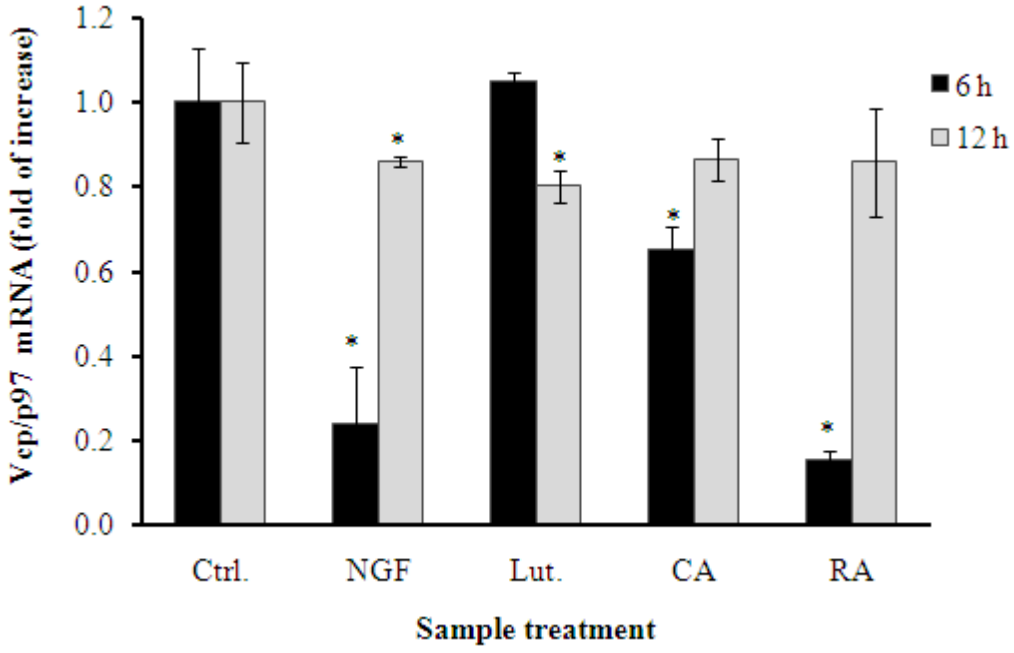
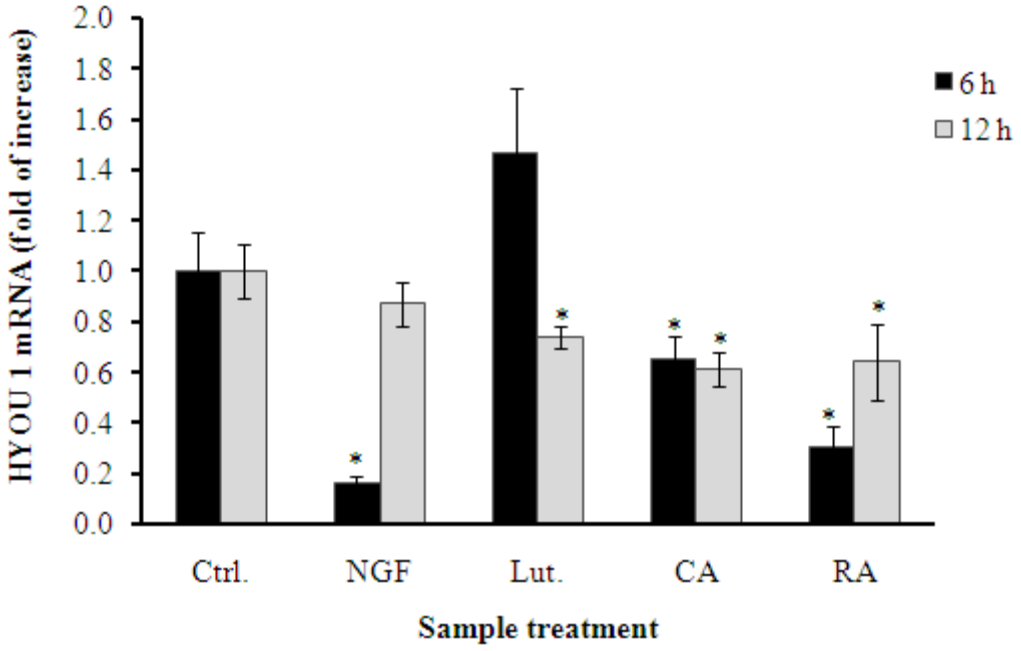

Vcp/p97 mRNA (fold of increase)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6 h
12 h
Ctrl.
NGF
Lut.
CA
RA
Sample treatment

Fig. 3

C


HYOU 1 mRNA (fold of increase)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6 h
12 h
Ctrl.
NGF
Lut.
CA
RA
Sample treatment

D

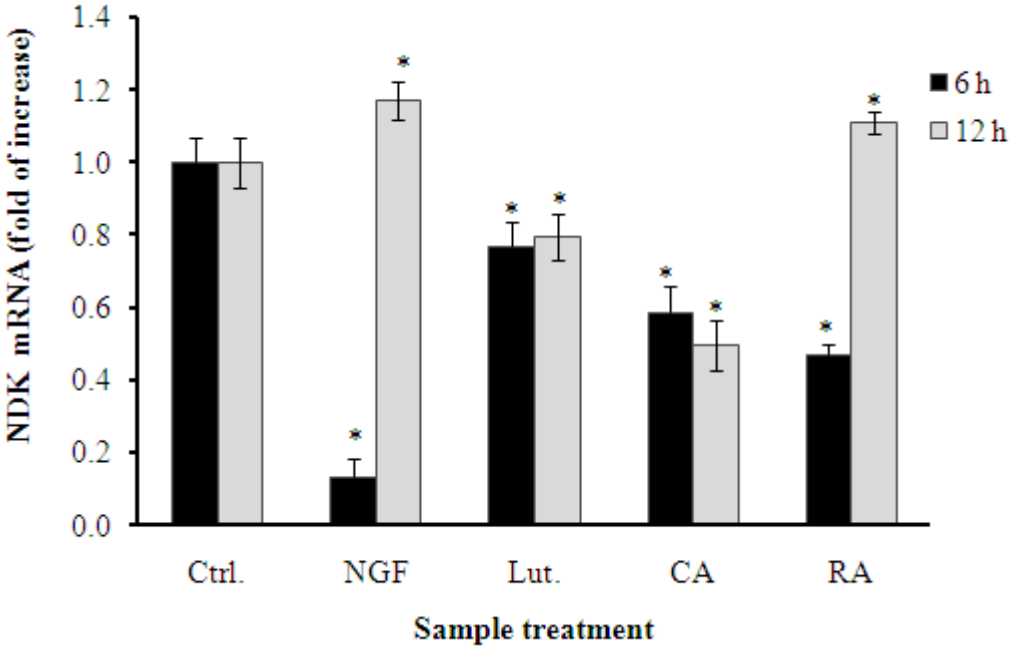

NDK mRNA (fold of increase)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
6 h
12 h
Ctrl.
NGF
Lut.
CA
RA
Sample treatment

Fig. 3

Table 1

Spot No	Accession No.	Score	Calculated *PI* value / Observed *PI* value	Calculated Mw value / Observed Mw value (KDa)	Protein sequence	Name of protein
1	P82995	80	4.93/ 4.0	85.161/ 100	K.VILHLKEDQTEYLEER.R	Heat shock protein alpha (***Hsp90α***)
2	P46462	454	5.14/ 5.5	89.977/ 105	K.MDELQLFR.G K.EMVELPLRHPALFK.A R.RIVSQLLTLMDGLK.Q R.EVDIGIPDATGRLEILQIHTK.N R.ETVVEVPQVTWEDIGGLEDVKR.E R.ELQELVQYPVEHPDKFLK.F K.GPELLTMWFGESEANVR. R.KYEMFAQTLQQSR.G	Transitional endoplasmic reticulum ATPase Valosin containing protein (***Vcp/p97***)
3	Q63617	639	5.11/ 5.5	111.448/ 115	R.SRFPEHELNVDPQR.Q R.SLAEDFAEQPIKDAVITVPAFFNQAER.R K.VLQLINDNTATALSYGVFR.R R.TLGGLEMELR.L R.DAVIYPILVEFTR.E R.YSHDFNFHINYGDLGFLGPEDLR.V K.LYQPEYQEVSTEEQREEISGK.L K.LCQGLFFR.V	Hypoxia up-regulated protein 1 (***HYOU1***)
4	P19804	313	6.92/7.2	17.386/ 23	R.TFIAIKPDGVQR.G K.DRPFFPGLVK.Y R.VMLGETNPADSKPGTIR.G R.GDFCIQVGR.N R.NIIHGSDSVESAEKEIGLWFKPEELIDYK.S K.EIGLWFKPEELIDYK.S	Nucleoside diphosphate kinase B (***NDK***)